

Video Article

Ordering Single Cells and Single Embryos in 3D Confinement: A New Device for High Content Screening

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Abstract

Biological cells are usually observed on flat (2D) surfaces. This condition is not physiological, and phenotypes and shapes are highly variable. Screening based on cells in such environments have therefore serious limitations: cell organelles show extreme phenotypes, cell morphologies and sizes are heterogeneous and/or specific cell organelles cannot be properly visualized. In addition, cells *in vivo* are located in a 3D environment; in this situation, cells show different phenotypes mainly because of their interaction with the surrounding extracellular matrix of the tissue. In order to standardize and generate order of single cells in a physiologically-relevant 3D environment for cell-based assays, we report here the microfabrication and applications of a device for *in vitro* 3D cell culture. This device consists of a 2D array of microcavities (typically 10^5 cavities/cm²), each filled with single cells or embryos. Cell position, shape, polarity and internal cell organization become then normalized showing a 3D architecture. We used replica molding to pattern an array of microcavities, 'egg cups', onto a thin polydimethylsiloxane (PDMS) layer adhered on a coverslip. Cavities were covered with fibronectin to facilitate adhesion. Cells were inserted by centrifugation. Filling percentage was optimized for each system allowing up to 80%. Cells and embryos viability was confirmed. We applied this methodology for the visualization of cellular organelles, such as nucleus and Golgi apparatus, and to study active processes, such as the closure of the cytokinetic ring during cell mitosis. This device allowed the identification of new features, such as periodic accumulations and inhomogeneities of myosin and actin during the cytokinetic ring closure and compacted phenotypes for Golgi and nucleus alignment. We characterized the method for mammalian cells, fission yeast, budding yeast, *C. elegans* with specific adaptation in each case. Finally, the characteristics of this device make it particularly interesting for drug screening assays and personalized medicine.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51880/>

Introduction

Current *in vitro* cell-based assays are two-dimensional (2D). This configuration is not natural for mammalian cells and therefore is not physiologically relevant¹; cells show a diversity of shapes, sizes and heterogeneous phenotypes. They present additional serious limitations when applied to screening applications, such as a disordered distribution within the plane and extreme phenotypes of cellular organelles (stress fibers, in particular). This is particularly important in clinical trials for drug testing, where high budgets are spent each year. Most of these drugs though fail when applied to animal models because of the artificial 2D culture condition in early stages of drug screening. In addition, by using this approach, specific cell organelles cannot be properly visualized, such as the cytokinetic actomyosin ring during cell mitosis, and generally structures that are evolving in the plane perpendicular to the plane of observation. Some new 2D assays have been proposed in order to overcome the above-mentioned drawbacks and important insights on cytoskeleton organization have been observed^{2,3}. However, these assays still present one serious limitation: cells show a very spread phenotype in contrast to what is observed *in vivo*, where cells present a 3D architecture. These artifacts associated with the culture method may trigger non-physiological features such as enhanced stress fibers^{1,4,5}.

Three-dimensional cell culture assays provide multiple advantages when compared to 2D environments^{6,7}. They are physiologically more relevant, and results are therefore meaningful. As an example, cells embedded in hydrogels show 3D-like structures but their morphologies differ from one cell to another^{8,9}. However, their morphologies differ from one cell to another, which complicates screening applications. An alternative strategy is to embed single cells in microfabricated cavities^{10,11}. Cell position, shape, polarity and internal cell organization can then become normalized. Besides providing 3D-like architecture to cells, microcavities also allows for high-content screening studies^{10,12-14}; single cells can be ordered into microarrays and cellular organelles and their evolutions can be observed in parallel. This regularity provides good statistics with low number of cells and better temporal/spatial resolutions. Useful compounds are easier to identify reliably.

In this study, we show the fabrication and application of a new 3D-like single cells culture system for high-content-screening applications^{10,12,13}. The device consists of an array of elastomeric microcavities (10^5 cavities/cm²), coined 'egg cups' (EC). Dimensions and total volume of EC in this work are optimized to the typical volume of individual NIH3T3 and HeLa cells during cell division. Morphology of the cavities – cylindrical

– is selected to properly orient cell shape for the visualization of active processes. Replica molding is used to pattern an array of EC onto a thin polydimethylsiloxane (PDMS) layer adhered on a glass coverslip^{15,16}. Cells are introduced in the EC by centrifugation. We report here observation and normalization of cellular organelles (actin stress fibers, Golgi apparatus and nucleus) in 3D (EC) in comparison with the same cells on 2D (flat) surfaces. We also report the observation of active dynamical processes such as the closure of the cytokinetic actomyosin ring during cell mitosis¹⁷. Finally, we show results of this methodology on other systems with rigid walls, such as budding yeast, fission yeast and *C. elegans* embryos which confirms the applicability of our methodology to a wide range of model systems.

We next present a detailed and exhaustive protocol in order to fabricate and apply the 'egg cups' for 3D microfabrication. Our approach is simple and does not need a clean room. We anticipate that this new methodology will be particularly interesting for drug screening assays and personalized medicine, in replacement of Petri dishes. Finally, our device will be useful for studying the distributions of cells responses to external stimuli, for example in cancer¹⁸ or in basic research¹⁹.

Protocol

1. Microfabrication of 'Egg cups'

1. Fabrication of the Master: Microcavities Array

1. Heat a 3" silicon wafer up to 200 °C to evaporate any presence of humidity.
2. Spin-coat a thin layer of SU-8 photoresist. Adjust the volume of resin and spinning speed depending on the desired thickness and photoresist type. This thickness will dictate the depth of the 'egg cups' (EC). For a 30 µm thick layer and SU-8 2025, spin-coat at 2,800 rpm.
3. Pre-bake the wafer at 65 °C for 1 min (step 1 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details.
4. Pre-bake the wafer at 95 °C for 3 min (step 2 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details.
5. Load the wafer on the mask aligner for UV exposure. Place the photolithography mask on it. The mask shows a pattern of circular features (disks) of 20 µm in diameter. Ensure a perfect contact between each other.
NOTE: Different manufacturers offer photolithography masks. The spatial resolution will determine the final cost. Acetate masks provide acceptable resolution (≈10 µm) at low cost. Chromium masks provide better resolution but are more expensive. Adapt the diameters of disks (from the photolithography mask) to the volume of cells. Dimensions of disks on the mask will determine the diameter of cavities in the device. Small diameters will lead to a low filling; too large diameter will not confine the cells. For HeLa and NIH3T3 cells, diameters of 20 µm to 25 µm are suggested.
6. Check the power of the UV lamp prior exposition and optimize the exposure time accordingly. Irradiate (wavelength = 365 nm) for 41.5 sec (or the optimized exposure time) at 250 mJ/cm².
NOTE: SU-8 2025 is a negative photoresist, which means that exposed regions to UV will be cured. In this case, the circular features were black and the rest transparent. Positive photoresist work in the opposite way: non-exposed regions are cured. Select the photoresist accordingly, depending on the design and photo-mask.
NOTE: Protect the eyes from UV light with appropriate safety glasses.
7. Remove gently the mask from the photoresist layer.
8. Post-bake the wafer at 65 °C for 1 min (step 1 of 2) for a 30 µm thick SU-8 2025 layer. Post-bake the wafer at 95 °C for 3 min (step 2 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details. After post-baking, cool the wafer to room temperature on the bench for around 1 min.
9. Place the wafer in the spin-coater and drop few mm of SU-8 developer to cover the whole wafer area. Develop for 2 min and then spin-coat at 1,000 rpm for 30 sec. Repeat the procedure three times.
10. Rinse with 2-propanol to ensure the complete removal of undeveloped SU-8. Appearance of white regions is an indication of incomplete development. If so, repeat the developing step an additional time.
11. Hard-bake the wafer at 200 °C to ensure robustness of the fabricated microstructures. This step is optional.
12. Store the 3" wafer with microstructures inside a 94 mm x 15 mm polystyrene Petri dish.
NOTE: There is no need for surface treatment, in particular silanization, for the next steps.

2. Fabrication of the Polydimethylsiloxane (PDMS) Replica: Pillars Array

1. Thoroughly mix in a 1:10 ratio (w/w) the cross-linker and the pre-polymer for a total of 30 g inside a 50 ml tube.
NOTE: Using a 1:10 (v/v) ratio is also working.
2. Centrifuge the tube at 1,800 x g for 5 min to remove air bubbles.
3. Drop gently the PDMS on top of the microstructures.
NOTE: If air bubbles appear during this step, degas the sample using a vacuum pump for 15-20 min.
4. Place the sample in an oven at 65 °C for 4 hr.
NOTE: The curing time varies between users and, together with the cross-linker:pre-polymer proportion. This time will dictate the rigidity of the PDMS. It is recommended to cure more than 2 hr and to stick to a fixed curing time.
5. Use a scalpel to gently cut the area of interest (stamp) of about 1 cm² which includes around 10⁵ microcavities or 'egg cups'.
NOTE: Cut first the PDMS and then, peel it off gently. Check the quality of the PDMS replica with an optical microscope.

3. Fabrication of 'egg cups' by replica molding. In the following, two alternative strategies for the fabrication of 'egg cups' are described. Both protocols are similar and provide identical results:

1. Strategy 1
 1. Activate the fabricated PDMS stamp by oxygen plasma treatment for 30 sec. Store temporarily the activated stamps into a closed Petri dish to prevent deposition of dust.
NOTE: Adjust the exposition time if other gases for the plasma are used.

2. Place the activated PDMS stamp upside up (the side with the structures) in a Petri dish next to a 15 ml tube cap. Fill the cap with 200 μ l of Trimethylchlorosilane (TMCS). Close the Petri dish and let the stamp silanize for 7 min.
NOTE: Some temporary deformation on the stamp and/or change in color (white) may be observed. The stamp will recover its original shape in short time and the structures will not be affected.
NOTE: TMCS produces acute inhalation and dermal toxicity, and is highly flammable (with ignition flashback able to occur across considerable distances). Consequently, it should be used in a fume cupboard away from sources of ignition.
 3. Place the PDMS stamp on the spin-coater with the structures upside up. Put a small drop of few microliters (around 20 μ l) of liquid PDMS (1:10 w/w cross-linker:pre-polymer) on top of the structures. Spin-coat at 1,500 rpm for 30 sec to deposit a thin layer of PDMS on top of the structures.
NOTE: If the stamp does not fit the spin-coater chuck place the stamp on top of a Petri dish lid with a small hole at its center.
 4. Place the stamp in the oven at 65 °C for 4 hr to cure the deposited spin-coated PDMS layer.
 5. Activate the thin PDMS layer by placing the PDMS stamp upside up, together with a glass coverslip #0 of 25 mm in diameter, using oxygen plasma cleaner for 30 sec. Proceed quickly to the next step.
NOTE: Coverslips with other thicknesses, shapes, and dimensions can be used as well. However, some cellular structures could be difficult to visualize depending on the selected coverslip thickness and objective magnification and/or NA and/or working distance. Check the objective data sheet.
 6. Place in contact the stamp (the side with the thin spin-coated layer) with the glass coverslip. Press gently all around the surface of the stamp with tweezers to make the 'bonding'. Finally, keep a constant pressure on top of the stamp for around 10 sec.
 7. After 30 min gently 'peel' the stamp out of the coverslip in order to 'liberate' 'egg cups' (see **Figure 1**). Rinse thoroughly with ethanol and dry. If PDMS 'egg cups' are not well adhered on the glass coverslip (*i.e.* they detach during the 'unpeeling' step), consider adjusting the settings of the plasma cleaner and restart at step 1.3.1.5.
NOTE: This step is delicate. Pay attention in order to avoid breakage of the coverslip and/or detachment of the thin PDMS layer.
 8. Glue a small piece (handle) of cured PDMS of 1 mm x 1 mm x 3 mm in volume at the edge of the coverslip with a small drop of liquid PDMS and cure the PDMS as before. This will facilitate the manipulation of the sample afterwards (see **Figure 1**). This step is optional.
2. Strategy 2
1. Hydrophilize the fabricated PDMS stamps by oxygen plasma treatment for 30 sec. Store temporarily the activated stamps in a closed Petri dish to prevent deposition of dust.
NOTE: Adjust the exposition time if other gases for the plasma are used.
 2. Hydrophilize the 25 mm diameter glass coverslips #0 oxygen plasma treatment at 15 W for 30 sec. Proceed quickly to the next step.
NOTE: Coverslips with other thicknesses, shapes, and dimensions can be used as well. However, cellular structures will be difficult to visualize depending on the selected coverslip thickness and objective characteristics (see note above).
 3. Spin-coat a small drop of PDMS (1:10 w/w cross-linker:pre-polymer) of few microliters onto the glass coverslips. Spin-coat at 1,500 rpm for 30 sec for a final thickness of around 50 μ m.
 4. Glue a small piece (handle) of cured PDMS of 1 mm x 1 mm x 3 mm in volume at the edge of the coverslip with a small drop of liquid PDMS and cure the PDMS as before. This will facilitate the manipulation of the sample afterwards (see **Figure 1**). This step is optional.
 5. Store temporarily the PDMS-coated coverslips onto a clean wipe inside a Petri dish to protect from dust deposition.
 6. Put a drop of silanizing reagent on top of each stamp and let it evaporate for 1-2 min. Then, dry them under a stream of N₂.
NOTE: In this step, a temporary deformation of the stamp can be observed during evaporation. The stamp will recover its original shape after drying with N₂ without any permanent deformation of microstructures.
 7. Drop very gently the silanized stamp on top of the PDMS-spin-coated glass coverslip stored in the Petri dish. Make sure that both sides are completely parallel during the contact. Avoid pressing or moving the stamp after placing it onto the PDMS-coated coverslip.
 8. Place the Petri dish with samples in the vacuum for 1-2 hr to remove air bubbles.
NOTE: Ensure that samples are totally horizontal to avoid stamp displacement. Avoid also vibration potentially caused by the vacuum pump.
 9. Place the samples in the oven at 65 °C for 4 hr.
 10. Gently, peel off the stamp to reveal 'egg cups'. Rinse thoroughly with ethanol and dry.
NOTE: Practice at this point is needed. Pay attention in avoiding breakage of the coverslip and/or detachment of the thin PDMS layer.

2. Introducing Cells into the 'Eggcups'

In order to introduce mammalian cells inside 'egg cups', PDMS surface needs to be functionalized with adhesion proteins of the extracellular matrix. This example uses fibronectin but other proteins of interest, such as collagen, could be used.

1. Hydrophilize the 'egg cups' in the oxygen plasma cleaner for 30 sec.
NOTE: Optimize the parameters if needed.
2. Prepare a solution in PBS 1x of 20 μ g ml⁻¹ fibronectin from Bovine sources.
3. Sterilize the 'egg cups' with UV for 5 min. Deposit a small drop (around 20-50 μ l) of fibronectin solution to cover the entire 'egg cups' area and incubate for 1 hr at RT. Protect the sample from drying.
4. Rinse gently the 'egg cups' with PBS 1x. Repeat 3 times.
NOTE: The sample is ready to use immediately or stored at 4 °C in the dark for several weeks.
5. Introduce a cylindrical custom-made plastic piece of 63 mm in height, 26 mm of external radius and 7 mm of internal radius dimensions into a 50 ml tube (see **Figure 2**)²⁰

CAUTION: Use UV-sterilized items or sterilize them prior use.

NOTE: This piece can be easily fabricated in the lab. or by any available machine shop.

6. Put 13 ml of cell culture medium inside the tube (see **Table 1**). The medium should fill at least 2 cm above the cylindrical piece to ensure complete immersion of the sample.

NOTE: For details of specific cell types, and other model systems such as yeast or *C. elegans* embryos, and the corresponding medium used, refer to section 5 and **Table 1**. The described protocol was optimized for HeLa, NIH3T3 cells, and other cell lines (see **Table 1**).

7. Introduce very gently the 'egg cups' inside the tube and parallel to the upper side of the plastic piece. Use sharp tweezers to hold the sample using the PDMS handle. Press gently the coverslip until it lies on top of the upper side of the plastic piece, until it is fully immersed (see **Figure 2**).

NOTE: It is recommended to use sharp and straight tweezers. With curved tweezers, the manipulation of the sample is difficult and may cause breakage.

8. Culture cells until 80-100% confluence in a P60 Petri dish and collect them by trypsinization.

NOTE: Cells can be wild-type, transfected or treated with any drug of interest.

NOTE: Avoid the formation of cell aggregates which will avoid single cells to enter the 'egg cups'. To optimize this step, pipette up and down thoroughly after trypsinization.

9. Re-suspend cells into 5 ml culture medium. Pipette 200 μ l of cells on top of the 'egg cups'.

NOTE: Drop cells as centered as possible on top of the 'egg cups' but avoiding physical contact. This will prevent breakage and/or damage of the sample.

10. Centrifuge at 1,800 x g for 2 min.

NOTE: After the first centrifugation, check in a microscope the filling percentage of the 'egg cups'.

11. Pipette again 200 μ l of cells on top of the 'egg cups' and centrifuge at 1,800 x g for 2 min. Repeat for a total of three times in order to optimize the filling percentage.

NOTE: After the last centrifugation, check with a microscope the filling percentage of 'egg cups'. If necessary, repeat the filling + centrifugation steps until reaching the desired filling percentage.

12. Remove the sample from the tube using the sharp tweezers holding the PDMS handle. Make sure to be careful in not 'disturbing' cells which are held in the 'egg cups' (see **Figure 2**).

13. Place the sample in a Petri dish with medium. Rinse to remove the excess of cells which are not in the 'egg cups' by pipetting up and down three times gently next to each side (total 4 sides) of the microstructure array.

NOTE: Pipetting too strongly may release some cells out from the 'egg cups'.

14. Replace the medium with fresh medium to remove nonattached cells.

NOTE: In this step a drug of interest can be added.

15. Fix cells or prepare them for time-lapse imaging. See step 4.1.

3. Observation of Active Cellular Dynamics in 'Egg cups': Cytokinetic Ring Closure

NOTE: This example uses HeLa cells which are transfected with MYH10-GFP and Lifeact-mcherry for myosin and actin, respectively, key active molecules involved in the cytokinetic ring closure during cell mitosis. The device is prepared with microcavities of 25 μ m in diameter. For their observation, an epifluorescence inverted microscope was used, equipped with a 60X oil objective (1.40 NA, DIC, Plan Apo) and GFP (myosin) and TxRed (actin) filters. Alternatively an upright confocal microscope was used, equipped with a 25X or 63X HCX IR APO L water objective (0.95 NA). For this example, it is highly recommended to synchronize cells by using the double thymidine block, mitotic block or mitotic shake-off method²¹⁻²⁴.

NOTE: The thickness of the PDMS used for the 'egg cups' allows the usage of a variety of objectives both in inverted and upright positioned microscopes.

1. Place 'egg cups' into a microscope holder and fill it with 1 ml of 10 % FCS L-15 observation medium. To avoid evaporation, place a glass lid on top of the holder or apply a thin layer of mineral oil on top of the medium. Select the 60X oil objective.

NOTE: L-15 medium is adequate for non-CO₂ atmospheres. Note also that some compounds of DMEM are auto-fluorescent. When using this medium, it is recommended to photobleach the fluorescent compounds by illuminating it with a high intensity lamp for 1-2 hr.

NOTE: Avoid using plastic lids when working with DIC imaging.

2. Place the holder with 'egg cups' and observation medium in the microscope. Focus carefully using brightfield light until the 'egg cups', and cells are in the same plane of observation.

3. Open the software and adjust the parameters. Select the filters TxRed and GFP for actin and myosin; adjust the exposition time for each channel. A typical acquisition rate is 5 sec for both channels.

NOTE: The exposition time may have to be adjusted depending on the setup used, dye or other cellular organelles of interest.

4. Select the region of interest and seek for a cytokinetic ring using either the GFP or TxRed channel. Focus accurately.

NOTE: The ring is sharper in myosin and easier to recognize.

5. Run the automatic acquisition in both channels until the ring is completely closed.

NOTE: Some photobleaching may be observed. Adjust the microscope parameters in order to reduce it.

4. Observation of Fixed Cellular Organelles into the 'Egg cups'

This step can be performed before or after step #3. Cells can be directly fixed after the centrifugation step and stained for the organelle of interest or after the observation in the microscope. This example shows the staining of the Golgi apparatus, nucleus and actin fibers on NIH3T3 fibroblasts in 'egg cups'.

1. Fixation of Cells in the 'Egg cups'

1. Prepare 3% paraformaldehyde (PFA) and warm at 37 °C. Remove the 'egg cups' sample from the 50 ml tube (or the microscope holder) and place it inside a P35 Petri dish. Rinse once with PBS 1x.

NOTE: Protocols for the preparation of 3 % paraformaldehyde are widely available elsewhere.

CAUTION: Use nitrile gloves and eye protection during the preparation of PFA.

- Remove completely the PBS and drop 1 ml of 3% PFA and incubate for 17 min. Remove the PFA and rinse twice with 1 ml of PBS 1X. Permeabilize cells using 1 ml of 0.5% Triton for 3 min and wash twice with PBS 1x for 5 min.

2. Staining of Cells in 'Eggcups'

- Incubate cells for Golgi apparatus staining with the primary antibody rabbit polyclonal anti-Giantin in a 1:500 dilution in PBS. Place a 100 μ l drop of antibody solution onto a plastic film sheet and incubate the cells inside the 'egg cups' upside down for 45 min.
NOTE: Protect the sample with a cover to prevent drying.
- Release carefully the 'Egg cups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1x.
- Prepare a cocktail in PBS with the secondary antibody Cy3 goat anti-rabbit (1:1,000) and with Phalloidin Alexa Fluor 488 (1:200) for staining actin stress fibers.
- Incubate cells with a 100 μ l drop of antibody solution onto a plastic film sheet and incubate cells inside the 'egg cups' upside down for 45 min.
- Release carefully the 'egg cups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1x.
- Incubate cells for nucleus staining placing a 100 μ l drop of 1 μ g ml⁻¹ DAPI in PBS onto a plastic film sheet and incubate cells inside the 'egg cups' upside down for 45 min. This step can be performed with step 4.2.3.
- Release carefully the 'egg cups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1x.
- Mount cells using a 15 μ l Glycerol:PBS (1:1 v/v) on a standard microscope glass slide and seal the sample with nail polish to avoid drying.
NOTE: Depending on the 'egg cups' thickness, mounting may be difficult. It is recommended to store then the sample into a P35 Petri dish in PBS, protected from drying.

3. Microscope Observation

NOTE: For this example an upright confocal microscope is used, equipped with PMT and Hybrid detectors. A 25X or 63X HCX IR APO L water objective (0.95 NA) was selected to provide a wide field of the sample and show the applicability of the device for high-content-screening applications.

- Select the 25X or 63X water objective.
NOTE: Different objectives can be used depending on the application and signal. But usage of high numerical aperture objectives is recommended.
- Place the fixed sample with the 'egg cups' and focus carefully using brightfield light (phase contrast or DIC) until the 'egg cups' and cells are in the plane of observation.
- Open the software and adjust the parameters. Select the filters GFP, Cy3 and DAPI for actin, Golgi and nucleus observation, respectively; adjust the exposition time for all channels.
NOTE: The exposition time may have to be adjusted depending on the setup.
- Select and focus the region of interest; start image capture (see **Figure 3**).

5. Adaptation for the Observation of Yeast Cells and *C. elegans* Embryo

1. Fission and Budding Yeast cells

NOTE: This example uses fission yeast cells which are tagged with RLC1-mcherry and CHD-GFP for myosin and actin, respectively. The budding yeast cells are not fluorescently labeled here. For fission yeast observation an inverted spinning disk confocal microscope was used. A 100X HCX PL APO CS oil objective (1.4 NA) was used for all acquisitions. Alternatively, cells were also observed using an inverted phase-contrast microscope equipped with a 20X phase contrast air objective LCPlanFI (0.4 NA). In this example, the protocol is identical for both cell types.

- Prepare the 'egg cups' surface as described above. For fission and budding yeast cells, prepare cavities of 5 μ m in diameter (see **Table 1**). In this case, the surface does not need to be functionalized with adhesion proteins.
NOTE: The filling can be optimized by using conical 'egg cups'. This shape captures and retains the cells avoiding releasing during the rinsing step after centrifugation. Filling percentage is optimum at about 80%. These conical 'egg cups' can be fabricated by means of Deep Reactive Ion Etching¹³.
- Culture yeast cells in the proper culture media (see **Table 1**) until reaching an optical density (OD) in the range of 0.2 and 0.8. Sonicate the culture of yeast cells to remove aggregates.
- Insert yeast cells in 'egg cups' by centrifugation. For centrifugation, 4 ml of cultured cells in the appropriate OD is added onto the tube with 'egg cups'. After the first centrifugation, gently shake the tube to re-suspend cells which are not in the 'egg cups', while cells in the 'egg cups' are not disturbed. Without opening the tube, centrifuge again and repeat this step twice. This ensures the deposition of cells from the culture into the empty microcavities and will increase the filling percentage.
NOTE: When working with yeast, it is recommended to pre-heat the centrifuge to the working temperature during experiments
NOTE: The protocol can be paused here and continued up to 12 hr later. In this case, store the sample at the working temperature and cover it to prevent evaporation.
- Place 'egg cups' in a microscope holder and fill the holder with filter sterilized medium for imaging. Now rinse the cells with the same media until the floating yeast cells are removed efficiently. Take care not to disturb cells in 'egg cups' during the rinsing process.
- Select the 100X oil objective and focus carefully. Open the software and adjust the parameters. For fission yeast, select the filters GFP and TxRed for actin and myosin and adjust the exposition time for both channels. A typical acquisition rate is 3 sec.
NOTE: Depending on the fluorophore, type of tagging and the set-up, exposure time varies for other systems.

2. *C. elegans* Embryo

NOTE: This example uses *C. elegans* embryos 25-30 μ m wide and 50-55 μ m long. Embryos were cultured as indicated in²⁵. A simple visual protocol of how to manipulate *C. elegans* can be found in²⁶. The observation was performed using an inverted phase-contrast microscope equipped with a 40X air objective 0.55 NA.

1. Prepare the 'egg cups' surface as described above and 25 μm in diameter (see **Table 1**). In this case, the surface does not need to be functionalized with adhesion proteins.
2. Culture the *C. elegans* embryos in the proper culture media (see **Table 1**).
3. Insert embryos in 'egg cups' by centrifugation as described above (see section 2.6 to 2.12) using ultrapure water as culture medium. NOTE: Embryos were 'behaving' normally in ultrapure water for the duration of the experiment. Alternatively use a physiological M9 buffer for long-term experiments.
4. Rinse the sample as described above (see section 2.14). Place the 'egg cups' into a microscope holder. Select the 40X air objective and focus carefully. Open the software and adjust the parameters. Select an acquisition rate of 3 sec.

Representative Results

The 'egg cups' (EC) are a novel high content-screening methodology which allows the visualization of oriented cells and embryos in a 3D environment. Additionally, some cellular processes, which are difficult to observe in standard 2D (flat) cultures, can be observed by this new method. **Figure 1a** shows a summary of the procedure for the EC microfabrication (see also Section 1 in the above-described protocol). The method is simple, fast, efficient and without any requirement of special equipment. **Figure 1b** and **1c** shows a large-scale picture and a magnified scanning electron microscope image of 'egg cups', respectively. As it can be observed, their shape and size are very regular. This method is very flexible; different shapes and sizes can easily be fabricated and adapted for different model systems. The dimensions of 'egg cups' were selected in the following manner: dimensions of cells which undergo division were measured on 2D surfaces: they have a spherical shape and their diameter was taken as a good indication for the EC diameter. Cells in 'egg cups' elongate and orient along their long axis during cell division for example. This dimension depends on the system – cells and embryos – so this dimension should be evaluated in each case.

Figure 2 shows the material needed (**Figure 2a**) and a step-by-step protocol (**Figure 2b**) about how to use the 'egg cups' (see also Section 2 in the above-described protocol). The filling of the EC with cells of interest (or other model systems) is very simple and fast. Typically, it takes less than 20-30 min, which also includes the time for cell trypsinization. After the filling, samples can be used to study active processes (live imaging) or can be fixed and stained for the visualization of organelles of interest (see also Sections 3 and 4 in the protocol described above).

On flat surfaces, cells show heterogeneous responses and extreme phenotypes of cellular organelles. In fact, it has been suggested that actin stress fibers (and other cellular organelles) are artifacts of the culture conditions¹. In order to prove this hypothesis, we cultured NIH3T3 cells both on 3D 'egg cups' and on flat surfaces and compared the phenotypes of different cellular organelles, namely actin stress fibers, Golgi apparatus and nuclei. **Figure 3** shows an example of how cells are organized on both configurations. In EC, cells are distributed in an ordered array showing a homogeneous spherical-like phenotype (**Figure 3a**). On flat surfaces, cells show the typical disordered, spread and heterogeneous morphology (**Figure 3b**). There are also significant differences in cytoskeleton structures. In particular, cells on 'egg cups' show a reduction in the number of stress fibers compared to flat surfaces. This is further confirmed in the 3D reconstructed images where no clear stress fibers are visible (see **Figure 3c-d**). This confirms that some cellular structures are magnified in 2D cultures. This is also in agreement with observations performed *in vivo* where stress fibers cannot be identified.

The Golgi apparatus also shows significant variation in their phenotype depending on the culture condition (see **Figure 4**). The Golgi apparatus on 2D cultures typically shows an extended phenotype 'embracing' the nucleus periphery whereas in 'egg cups' it shows a more compacted phenotype (see **Figure 4a-b**). In order to simulate a drug screening manipulation, we also evaluated the effect of drugs on cells cultured on both environments. We selected Blebbistatin mainly because it disrupts the actin stress fibers and could have an effect on Golgi morphology (see **Figure 3c-d**). Since the Golgi is located next to the cell nucleus, this drug could also have an effect on its architecture. We first observed that cells treated with this drug showed a less regular and uniform morphology compared to wild type (WT) cells (see **Figure 3c-d**). We then compared and quantified the Golgi phenotype observed on 'egg cups' and on flat surfaces (see **Figure 4c**). We observed that on 2D surfaces cells showed mostly an extended phenotype whereas on 'egg cups' cells showed a more compacted phenotype. We did not observe though a striking difference between WT and Blebbistatin-treated cells.

Finally, on 2D surfaces the cell nucleus is randomly oriented whereas for cells in EC it is orthogonally oriented with respect to the XY plane in both WT and Blebbistatin treated cells (see **Figure 5a-c**). This highlights the strength of the device to orient cellular organelles, similar to a former application of the method for orienting the plane of observation of the cytokinetic ring in yeast and mammalian cells^{10,12,13}. We finally studied how the nucleus sphericity (defined as $\psi = [\pi^{1/3} 6V_n^{2/3}]/A_n$, where V_n is the volume of the nucleus and A_n its surface area) was affected depending on the culturing condition and upon the treatment of cells with Blebbistatin. **Figure 5d** shows the corresponding distributions of ψ . We did not observe a difference for WT^{flat} vs WT^{EC} , which reveals that the EC are not affecting the normal sphericity of cells. However, we observed a difference when comparing WT^{EC} to $Bleb^{EC}$ suggesting that the EC are revealing a real effect of the drug that is masked in 2D.

Live cell studies using 'egg cups' allow also identification of novel active processes which are not visible in standard cultures. We plated cells in EC and visualized cell division. **Figure 6** shows a sequence of images of the cytokinetic ring closure during cell mitosis. The 'egg cups' device allows a complete visualization of the ring, whereas standard 2D cultures only shows two areas which corresponds to one single plane¹⁰. Reconstruction of the ring from a sequence of z-stack images using 2D cultures can be done²⁷, but important information is lost. The quality is diminished due to low z resolution and dynamic processes cannot be resolved. Actin and myosin are the key proteins in the force generation of cell division. Their dynamics cannot be imaged and studied in 2D culture (**Figure 6a**), whereas with 'egg cups' it is immediately revealed. We have identified novel structures and processes: in HeLa cells we find periodic accumulations of myosin¹⁷. These accumulations move radially as the ring is closing (**Figure 6b**). In fission yeast we also find inhomogeneities in myosin and actin (**Figure 6c, right**)¹⁷. In contrast to what we see in HeLa cells, they rotate on the ring during closure. The speed is in the range of $\mu\text{m min}^{-1}$ and would not be resolvable by z reconstruction with standard microscopes. Finally the cytokinetic ring can be further studied by staining for its components. We find that there is an accumulation of phosphotyrosine in the vicinity of the ring (**Figure 6d**). We can also show that anillin is colocalizing in the ring (**Figure 6e**). By staining the cells in this orientation, we reveal that anillin shows also an inhomogeneous distribution.

The 'egg cups' were also applied to different model systems: we reported mammalian cells, fission yeast, but we also tested budding yeast and *C. elegans* (see **Figure 7a-e**). In this case, the protocol was adapted for each specific system in terms of culture media, cavities size and

morphology (see **Table 1**). As an example, conical V-shaped 'egg cups' were the optimal morphology for immobilizing fission yeast efficiently¹², instead of completely cylindrical (or U-shaped) shape used for mammalian cells¹³. This allowed testing the effect of different cytoskeleton drugs with potential application in Life Science research. This demonstrates the flexibility and reliability of the developed methodology.

Furthermore, the highly ordered arrangement of cells allows an easy, automated read-out of the fluorescence of single cells. We illustrate this by inserting NIH3T3 cells expressing GFP in 'egg cups' (**Figure 8a**). The cell position can be easily recognized and the corresponding expression level measured. **Figure 8b** shows the distribution of fluorescence signals. This can be applied to any read-out (immunofluorescence, fluorescent reporters in cells for example).

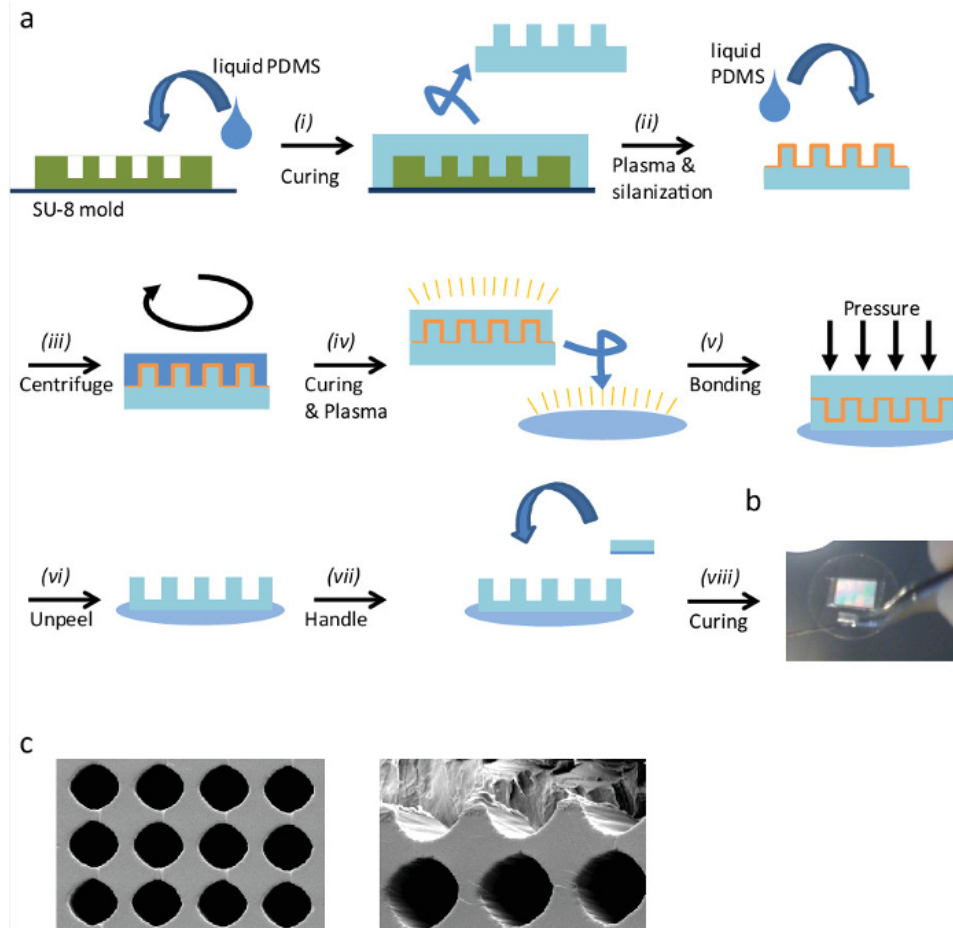


Figure 1: Fabrication of 'egg cups'. (a) Schematic description of the fabrication procedure of 'egg cups' by replica molding: (i) Pour liquid PDMS on the SU-8 mold and cure it. (ii) Cut out the stamp and remove it carefully from the surface, then plasma activate it to silanize it. (iii) Pour liquid PDMS on the silanized stamp and centrifuge it to obtain a thin PDMS layer. (iv) After curing the PDMS layer, plasma activate both, the PDMS covered stamp and a glass coverslip. (v) Plasma bind both by applying a gentle, homogeneous pressure. (vi) After plasma bonding, remove carefully the stamp to uncover the 'egg cups' surface. (vii) To simplify the handling in the next steps, add a small PDMS handle piece. Bind the PDMS piece to the coverslip by gluing it with liquid PDMS and (viii) cure it then in the oven. (b) Image of a 25 mm coverslip with PDMS 'egg cups' and a handle. (c) Scanning electron microscope images of PDMS 'egg cups'. The distance between centers of 'egg cups' is 30 μm , and their diameter about 25 μm . (Left) Top view. (Right) 'Egg cups' are cut to image the inner part. [Please click here to view a larger version of this figure.](#)

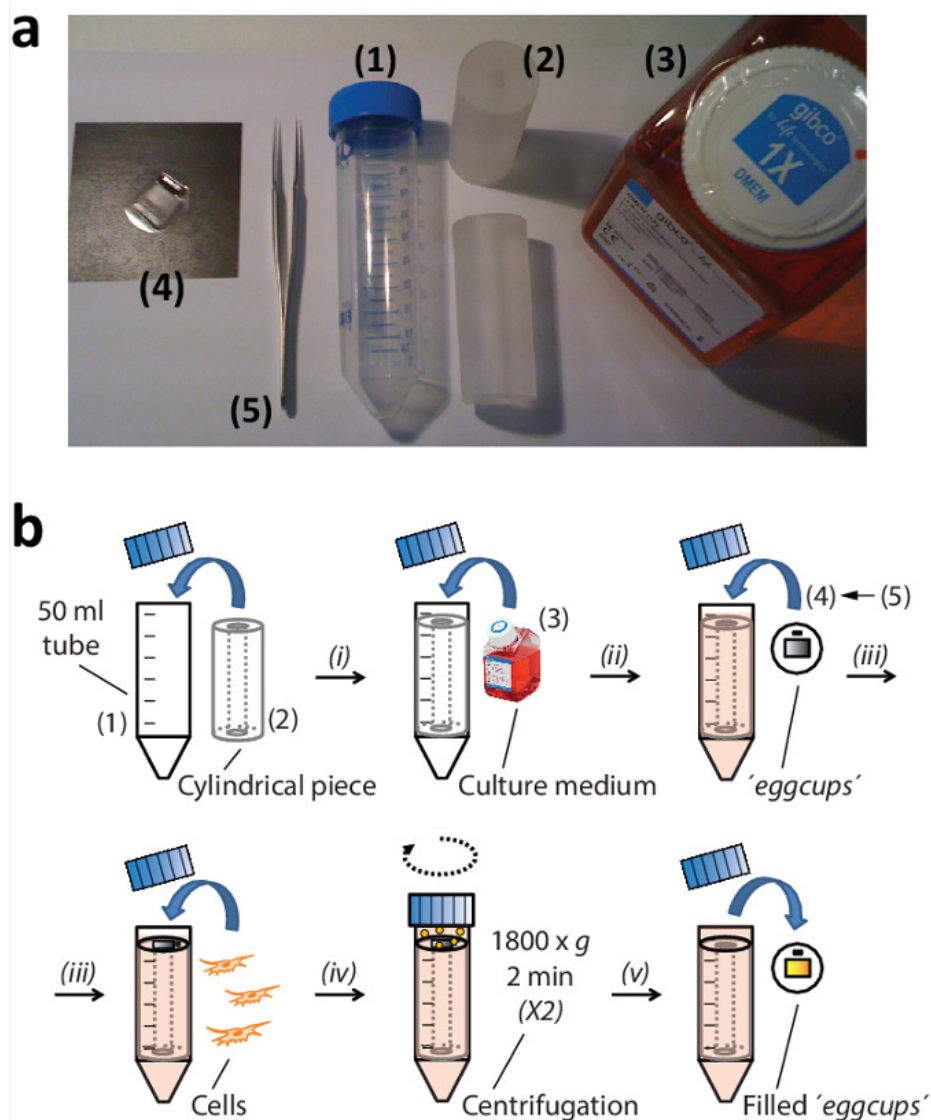


Figure 2: (a) Elements needed for the EC filling. (1) 50 ml tube; (2) cylindrical piece (top and side view); (3) cell culture medium; (4) 'egg cups'; (5) sharp tweezers. (b) Schematic of the EC filling procedure. (i) A cylindrical piece is first introduced into a 50 ml tube and filled with 13 ml of cell culture medium. Next, (ii) the 'egg cups' are gently deposited on top of the cylindrical piece using sharp tweezers to manipulate the EC using the small PDMS piece. (iii) Cells at the proper density are pipetted on top of the EC. (iv) Cells are introduced in the 'egg cups' by centrifugation. (v) Finally, the sample is gently released out from the tube and it is ready to use. [Please click here to view a larger version of this figure.](#)

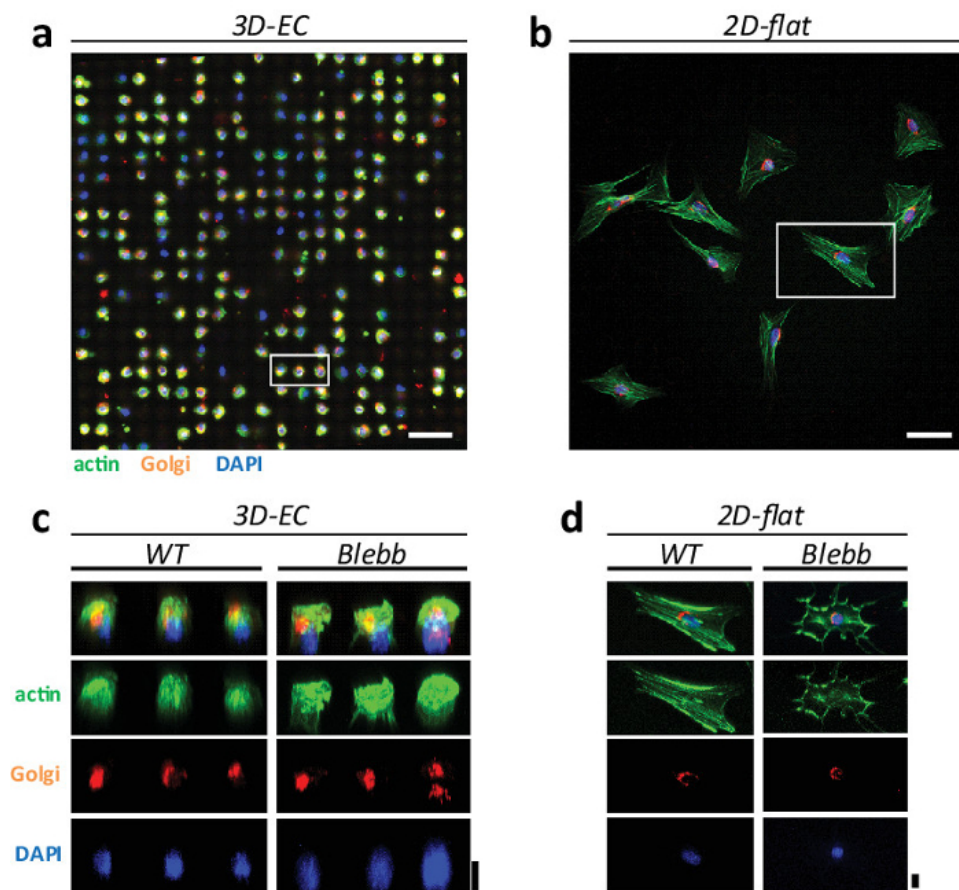


Figure 3: Comparison of cell phenotypes on 3D 'egg cups' and 2D flat surfaces. Confocal microscopy (25X water objective, 0.95 NA, Leica) image of NIH3T3 cells on (a) EC forming an ordered array, and showing a homogeneous spherical phenotype, and on (b) standard 2D flat culture, randomly distributed with heterogeneous phenotypes. Cells were stained for actin (in green), Golgi (in orange) and nucleus (in blue). Scale bars = 100 μm. (c) 3D reconstruction of cells on EC and (d) on flat surfaces for WT and Blebbistatin-treated cells. Scale bars = 20 μm. [Please click here to view a larger version of this figure.](#)

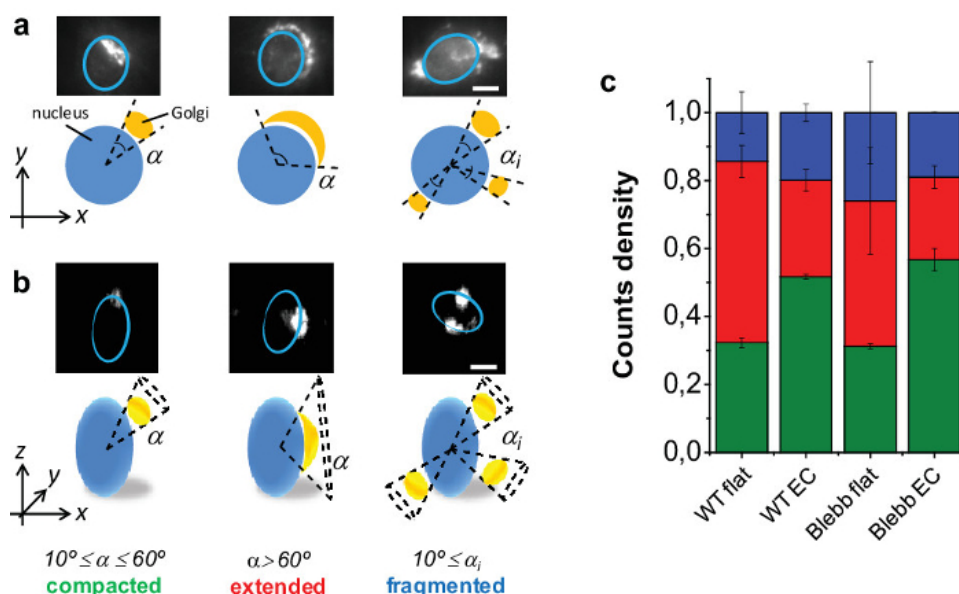


Figure 4: Study of NIH3T3 Golgi apparatus phenotype. Schematic and sample image of Golgi phenotype classification for cells on (a) flat and (b) EC. Cells were classified as compacted, extended or fragmented depending on the α -value. (c) Quantification of Golgi phenotypes. Scale bars = 10 μm. [Please click here to view a larger version of this figure.](#)

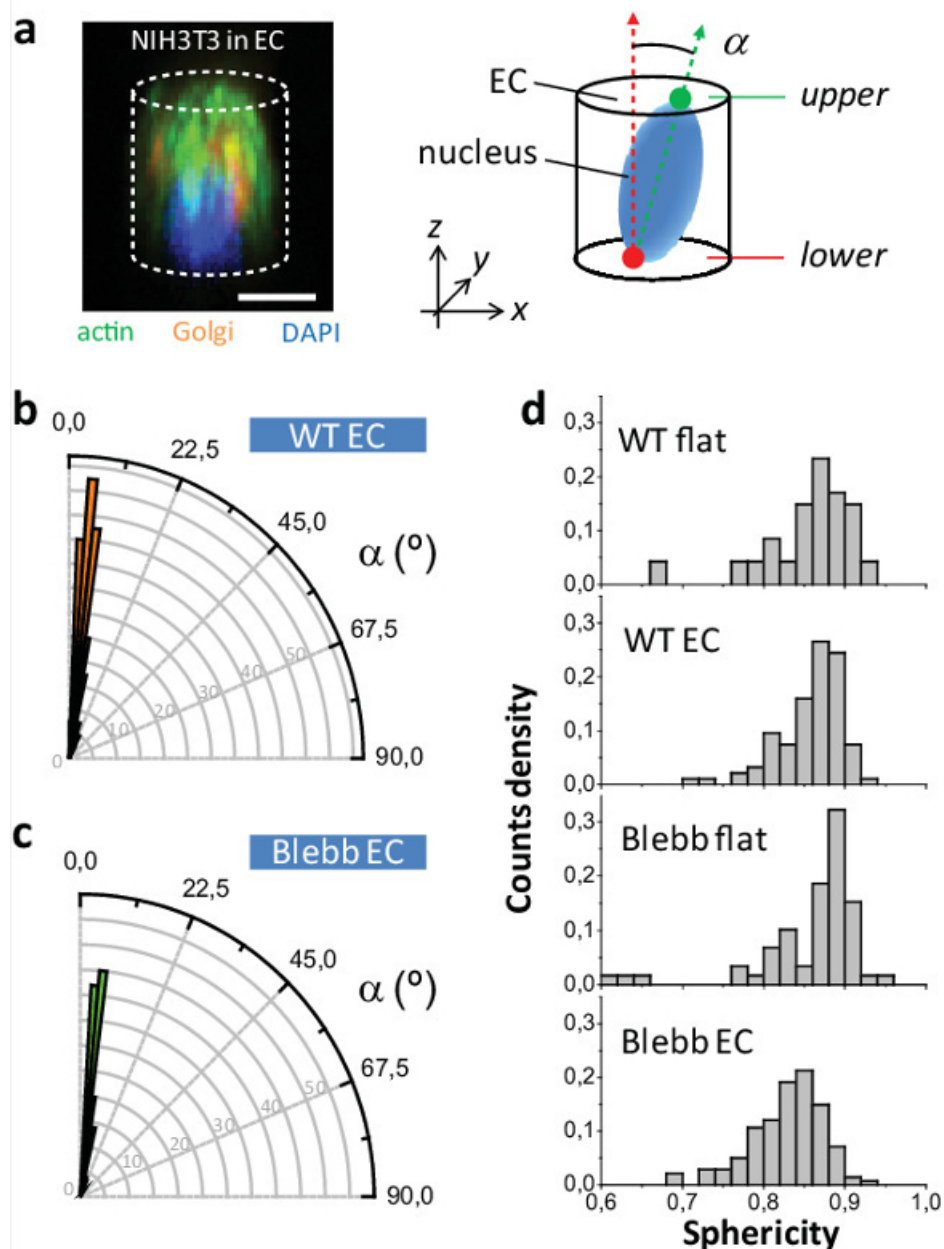


Figure 5: Study of NIH3T3 nucleus phenotype. (a) (Left) Confocal microscopy image of a NIH3T3 cell inside an EC and stained for actin (in green), Golgi (in orange) and nucleus (in blue). (Right) Scheme of nuclei orientation inside EC. (b) Angular distribution of nuclei inside EC for WT and (c) Blebbistatin-treated cells. (d) Nucleus sphericity values for WT and Blebbistatin-treated cells both for EC and flat surfaces ($P[\text{WT}^{\text{EC}} - \text{Blebb}^{\text{EC}}] < 0.001$, $P[\text{Blebb}^{\text{flat}} - \text{Blebb}^{\text{EC}}] < 0.000.1$; $n_{\text{WT}^{\text{flat}}} = 47$, $n_{\text{WT}^{\text{EC}}} = 94$, $n_{\text{Blebb}^{\text{flat}}} = 59$, $n_{\text{Blebb}^{\text{EC}}} = 141$ cells). Scale bar = 10 μm . [Please click here to view a larger version of this figure.](#)

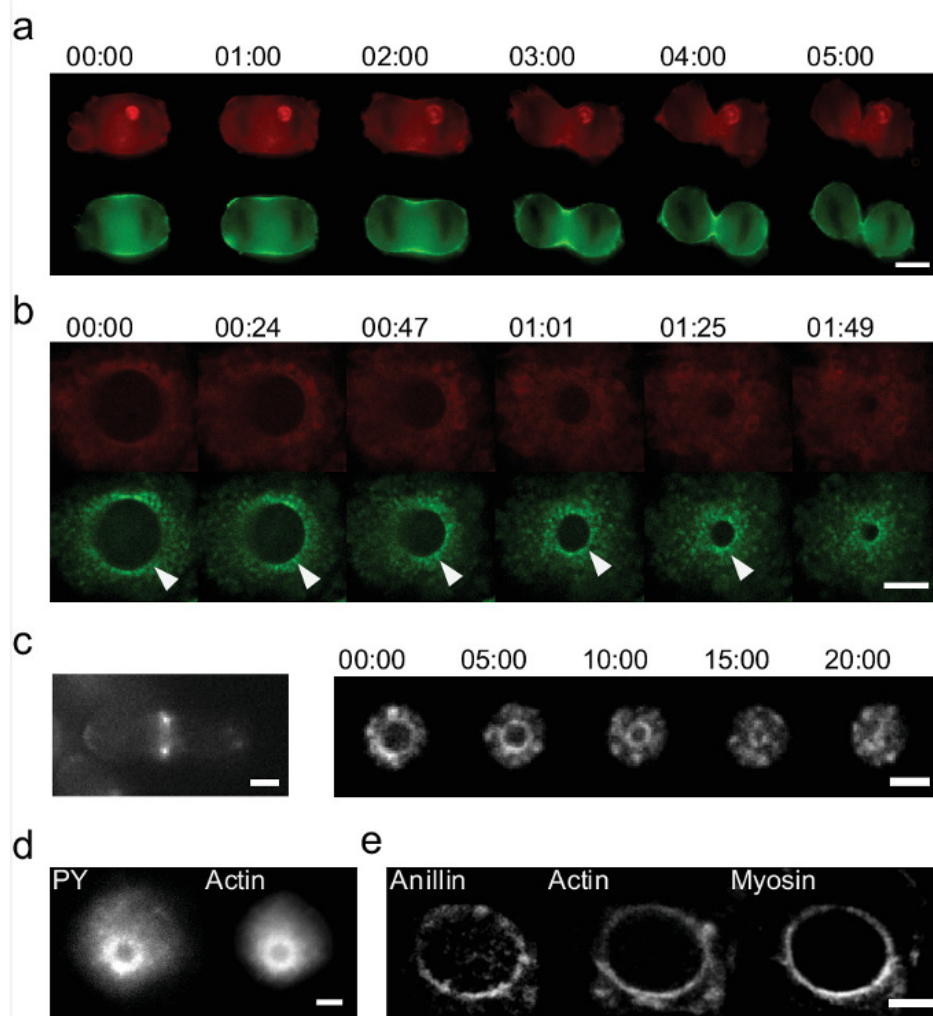


Figure 6: Detailed study of the cytokinetic ring in live and fixed samples and in two systems using 'egg cups'. (a) Time sequence of the cytokinetic ring using standard 2D *in vitro* culture. Only two bright spots in actin (Lifeact-mcherry, red) and myosin (GFP tagged, green) are visible in the cleavage furrow of the HeLa cells (Scale bar = 10 μ m). (b) Time sequence of the closure for the cytokinetic ring in HeLa cells during mitosis using 'egg cups'. The images show actin (in red) and myosin (green). 'Egg cups' allow the identification of still myosin accumulations. One example is highlighted with an arrowhead. (Scale bar = 5 μ m). (c) The cytokinetic ring can also be visualized in fission yeast. (Left) Cells lie on a flat surface, the cytokinetic ring is only visible as two dots. (Right) Cells in 'egg cups': the entire closure can be captured. Actin is labeled with CHD-GFP (Scale bars = 2 μ m). Time in min:sec. (d-e) Examples of stained cytokinetic rings. (d) Actin-GFP expressing HeLa cells are stained for phosphotyrosine (PY) which also shows signal in the ring (Scale bar = 5 μ m). (e) HeLa cells expressing GFP tagged myosin and Lifeact-mcherry (actin) are stained for anillin. Anillin is revealed to localize in the cytokinetic ring and less concentrated in the cortex. It shows co-localization with actin and myosin (Scale bar = 5 μ m). [Please click here to view a larger version of this figure.](#)

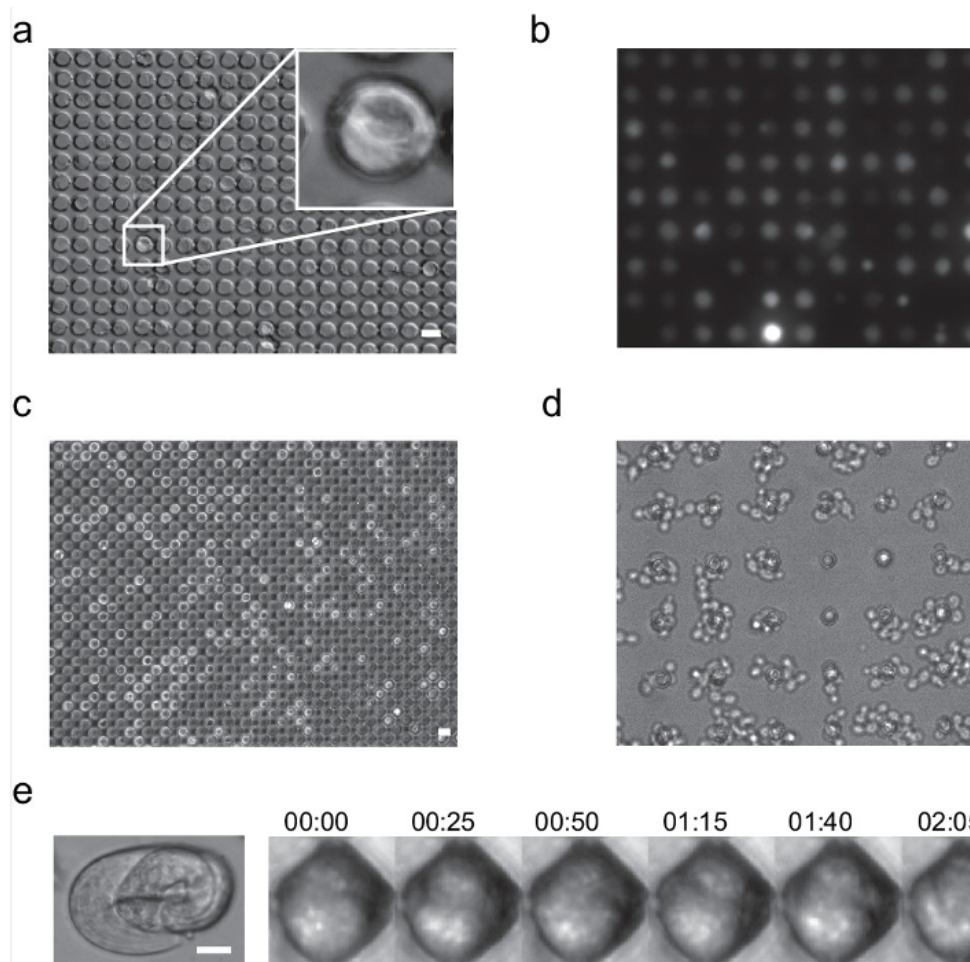


Figure 7: Application of the 'egg cups' to other cell types and model systems. (a) U2OS (human osteosarcoma). The inset shows a dividing cell. (Scale bar = 20 μ m). (b) NIH3T3 cells expressing GFP. Difference in expression levels can be easily read out (Scale bar = 20 μ m). (c) SW480 cells (Scale bar = 20 μ m). (d) Budding yeast; their cycle time is unchanged. (Scale bar = 10 μ m). (e) *C. elegans* worms; (Left) on a flat surface. (Right) In 'egg cups', embryo is seen from an otherwise hidden perspective. (Scale bars = 10 μ m). Time in min:sec. [Please click here to view a larger version of this figure.](#)

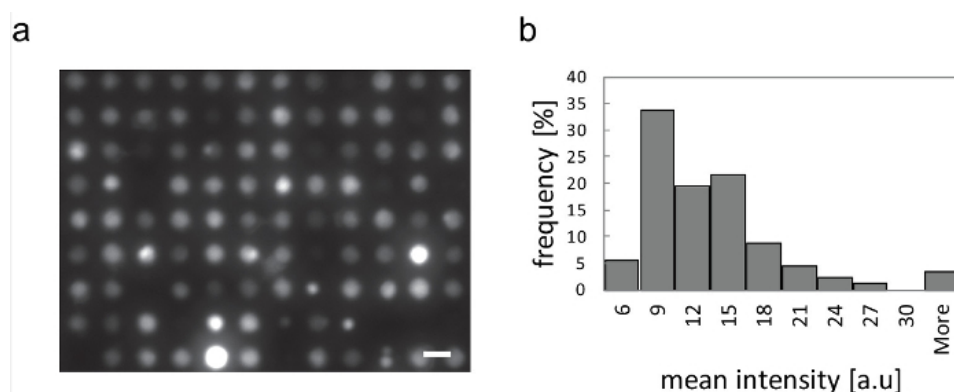


Figure 8: The organization in an array of 'egg cups' allows an automated analysis of cell population. (a) NIH3T3 cells in EC (Scale bar = 20 μ m). They have different expression levels of GFP. (b) Automated recognition of cell position allows an individual analysis of the expression level. It is summarized in the histogram of the GFP expression of the cell population. [Please click here to view a larger version of this figure.](#)

Model System	Type	Culture Medium	Observation Medium	'eggcup's' diameter (μm)	Comments/Description
Mammalian cells	NIH3T3	10 % BCS high-glucose DMEM	10 % BCS L-15	20	Other stable cell lines, such as REF52 or MDCK, as well as primary cell lines, cancerous cells and/or stem cells can also be inserted in the 'eggcup's'.
	HeLa	10 % FCS high-glucose DMEM	10 % FCS L-15	25	Available from many different sources.
	U2OS	10 % FCS high-glucose DMEM	10 % FCS L-15	20-25	Available from many different sources.
	SW480	10 % FCS high-glucose DMEM	10 % FCS L-15	17-20	Available from many different sources.
Yeast	Fission Yeast	Agar plate (YE5S) and liquid media (YE5S and EMM5S)	Filter sterilized EMM media (see the list of materials)	5	The surface does not need to be functionalized with adhesive proteins.
	Budding Yeast	Agar plate (YPD) and liquid media (YEPD and SD)	SD media	5	The surface does not need to be functionalized with adhesive proteins.
Embryo	<i>C. elegans</i>	NGM plate	ultrapure water	25	Alternatively M9 medium can be used for long-term experiments. The recipe of this salted solution can be found here: http://cshprotocols.cshlp.org/content/2009/5/pdb.rec11798.full?text_only=true

Table 1: Culture conditions in 'eggcup's' for different model systems. The above-related protocol can easily be adapted by just replacing the described culture conditions and the size of 'eggcup's'.

Discussion

Replica molding was used in order to fabricate the 'eggcup's'. The fabrication process does not need a clean room; it is easy and simple, although some practice may be required. In particular, releasing the PDMS stamp is the most critical step in order to produce a large area of high quality 'eggcup's'. For this reason, special care has to be taken in this step. If this step is repeatedly failing, consider to optimize the plasma cleaner parameters prior to the silanization and plasma binding. Insufficient silanization will lead to strong sticking of the stamp to the PDMS film. If this is observed, the incubation time with the silanizing reagent can be increased. Note that other techniques and materials can be applied to fabricate the 'eggcup's', which can be functionalized with a large range of ligands (fibronectin, gelatin, collagen, *etc.*). In particular, microcavities in polystyrene can be easily fabricated by custom-made hot-embossing technique. This ensures biocompatibility and direct comparison with results obtained in standard culture dishes. Similarly, special care and practice are required in order to optimize the filling percentage. In particular, the rinsing step is critical in order to ensure an appropriate filling with no excess of cells, contributing to noise and background in the signal. If cells are removed easily from cavities, consider to change the size or depth of cavities.

'Eggcup's' provide 3D-like architecture to cells and high-content screening assays using a simple protocol. Cellular organelles and active processes unknown using standard culture assays can be easily visualized by means of inserting single cells on individual microcavities ('eggcup's'). Depending on the model system, the size, shape and their dimensions can be easily adapted. In this way mammalian cells, fission yeast, budding yeast and *C. elegans* can be manipulated and studied, as well as any embryos such as *Drosophila*, mice or human embryos for *in vitro* fertilization, or stem cells for example.

In this setup single cells are captured. This is in contrast to epithelial tissues encountered *in vivo*. However, this environment could be reproduced in our 'eggcup's' by coating the side walls with cadherins to mimic cell-cell contacts using more flexible elastomers. Focal contacts will be promoted by the deposition of fibronectin at the bottom of wells. These respective distributions of adhesion molecules should allow in reproducing the cellular environments encountered *in vivo*. By this method one would approach the physiological conditions.

Medium exchange in our assay is ensured. Cells in EC do not show any degradation when performing both short- and long-term experiments due to lack of medium exchange. Note also that cells in EC can be cultured until confluence although the main interest is when individual cells or embryos are isolated within the cavities.

Orientation of organelles or entire organisms is revealing new information. We show different dynamics of actin and myosin in the cytokinetic ring. Although the cytokinetic ring in fission yeast and mammalian cells is composed of similar key components, we show with this setup, that their specific dynamics is different¹⁷. This is supporting the result, that the closure mechanism in the two systems is different as well. To develop and investigate such a hypothesis, the orientation of the cell is indispensable. In future studies, this device can be also used to investigate other events related to organelle organization in cells.

Beyond that, this technique can be of great use in developmental biology. Elongated embryos can be easily oriented, observed or further treated in a defined orientation. Probably our assay would not impose polarity of embryos, but the high filling percentage would allow to extract the desired read-out in a reliable manner. Altogether 'egg cups' could be a good device for high-content screenings.

Other culture assays have been proposed. These methods range from multiple cells in 2D dimensions in multiwell plates, to single cells deposited in micropatterned adhesive motifs with identical shape. However, none of them is appropriate to overcome the limitations detailed above on the observation of cellular organelles and dynamical processes¹.

Future improvements to our system will allow the applicability of 'egg cups' to industry-oriented purposes. As an example, drug screening applications in pharmaceutical companies require the use of multiwell plates^{14,28}, implementing 'egg cups' into such platforms will potentially improve the reliability of tests and results. As such, high content-screening assays will be performed using the commonly used automatized processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability. Some commercial products based on 3D-cell cultured assays have already appeared in the market highlighting the importance of this kind of assays. Finally, these devices open new perspectives for personalized medicine: cells from patient could be placed in 'egg cups', and treatment cocktails could be tested in a physiological environment; the biomarker read-out will allow to anticipate an optimal treatment to be given to the patient²⁹. Altogether the physical shape of the cells and embryos are guiding the architecture of the cavities, and we hope that the device and this method will be widely spread in the future.

Disclosures

We have nothing to disclose.

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